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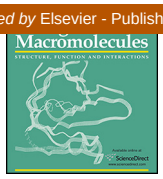

Purification and primary structure of a novel mannose-specific lectin from *Centrolobium microchaete* Mart seeds

Mayron Alves de Vasconcelos[a,*], Ana Cecília Alves[b,2], Rômulo Farias Carneiro[c,2], Artur Hermano Sampaio Dias[b,2], Francisco William Viana Martins[b,3], João Batista Cajazeiras[b,2], Celso Shiniti Nagano[c,3], Edson Holanda Teixeira[a,1], Kyria Santiago do Nascimento[b,2], Benildo Sousa Cavada[b,**,2]

[a] *Laboratório Integrado de Biomoléculas–LIBS, Departamento de Patologia e Medicina Legal, Universidade Federal do Ceará, Monsenhor Furtado, s/n, 60430-160, Fortaleza, Ceará, Brazil*

[b] *Laboratório de Moléculas Biologicamente Ativas - BioMol-Lab, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Campus do Pici, s/n bloco 907, 60440-970, Fortaleza, Ceará, Brazil*

[c] *Laboratório de Biotecnologia Marinha–BioMar-Lab, Departamento de Engenharia de Pesca, Universidade Federal do Ceará, Campus do Pici s/n, bloco 871, 60440-970, Fortaleza, Ceará, Brazil*

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ABSTRACT

This study aimed to purify and characterize a novel mannose-binding lectin from the seeds of *Centrolobium microchaete*. *Centrolobium microchaete* lectin (CML) was purified by affinity chromatography in mannose-Sepharose-4B column. CML agglutinated rabbit erythrocytes and was inhibited by D-mannose, α-methyl-D-mannoside, D-glucose, N-Acetyl-D-glucosamine and sucrose. The lectin was stable at pH 7.0 and 8.0 and temperatures up to 60 °C. The monomeric form of CML showed approximately 28 kDa, and its native form is probably a homodimer, as determined by gel filtration chromatography. The primary structure of CML was determined by tandem mass spectrometry that showed CML as a protein with two distinct forms (isolectins CML-1 and CML-2) with 246 and 247 residues, respectively. CML-2 possesses one residue of Asn more than CML-1 in C-terminal. The primary structure of CML agrees with the molecular weights found by electrospray ionization mass spectrometry: 27,224 and 27,338 Da for CML-1 and CML-2, respectively. CML is a metal-dependent glycoprotein. Moreover, the glycan composition of CML and its structure were predicted.

1. Introduction

Lectins are proteins, or glycoproteins, with at least one carbohydrate or derivate binding site without catalytic function (nonenzymatic) or immunological characteristics [1], widely distributed in nature in organisms such as in viruses, fungi, bacteria, animals and plants [2]. Plant lectins are the most studied since they are present in different plant tissues and show different functions, structures, and carbohydrate-binding specificities [3]. Furthermore, plant lectins have already demonstrated their potential as tools in the identification, purification, and stimulation of specific glycoconjugates [4]. Lectins have also been used as tool to distinguish between cell types, and are involved in several biological activities as mitogenic [5], antimicrobial [6], anti-inflammatory [7], anti-insect [8] and antitumor [9] activity.

Plant lectins were classified into seven families according to their evolutionary and structural characteristics [3], and among these families, lectins of the Leguminosae family are, thus far, the most studied group. In fact, several legume lectins having already been isolated and characterized in terms of their chemical, physical, structural and biological properties [10].

Legume lectins show a remarkable conservation in primary, secondary, and tertiary structures [10]. These proteins consist of two or more subunits of 25 to 30 kDa and show a wide variation

* Corresponding author at: BioMol-Lab, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Campus do Pici, s/n bloco 907, 60440-970, Fortaleza, Ceará, Brazil. Tel.: +55 85 33669818; fax: +55 85 33669818.

** Corresponding author at: LIBS, Departamento de Patologia e Medicina Legal, Universidade Federal do Ceará, Monsenhor Furtado, s/n, 60430 160, Fortaleza, Ceará, Brazil. Tel.: +55 85 33668300.

E-mail addresses: mayronvasconcelos@gmail.com (M.A.d. Vasconcelos), bscavada@gmail.com (B.S. Cavada).

[1] Tel: +55 85 33668300.

[2] Tel.: +55 85 33669818.

[3] Tel.: +55 85 3366972.

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of sugar-binding affinities, despite high similarity of amino acid sequences among family members. Interaction with carbohydrates requires tightly bound metal ion [11,12].

Interestingly, studies with legume lectins are focused on the subfamily Papilionoideae with a few investigations of lectins from the most primitive tribes, such as Swartzieae, Sophoreae and Dalbergieae [13–15]. Some lectins from the Dalbergieae tribe have been isolated and biochemically characterized; among these are galactose-binding lectins [13,16,17] and mannose-binding lectins [18,19]. Moreover, two recombinant mannose-binding lectins have been structurally characterized: lectins from *Pterocarpus angolensis* (PAL) and *Platypodium elegans* (PeLa) [15,20]. Regarding primary structure of Dalbergieae lectins, few studies have been conducted. So far, the primary structure of PAL, PeLa and of lectins from *Vatairea macrocarpa* and *Vatairea guianensis* were determined [13,15,20,21]. Furthermore, the amino acid sequence of the lectins isolated from *Platymiscium floribundum* and *Centrolobium tomentosum* has been partially determined [18,19].

Centrolobium, Dalbergieae tribe [22], is a neotropical genus of seven species. It can be easily recognized, even when sterile, by an abundance of orange peltate glands covering most parts [23]. The genus *Centrolobium* is found in parts of Brazil, Bolivia, Ecuador, Peru, Colombia, Venezuela, Panama, and the Guianas. *Centrolobium* shows a mixture of species distribution patterns, with both widespread species and more geographically restricted endemics [23]. *Centrolobium microchaete* occurs naturally in Brazil and is usually known as “araribá-amarelo”, “pitimuju” and other popular names [24]. As a consequence of the strength and attractive appearance of the wood from *C. microchate*, it is suitable for making furniture, and tinctures are extracted from bark and roots [24]. Studies involving this genus are limited to investigations of phenolic compounds [25,26], and only one lectin has been reported recently: *Centrolobium tomentosum* lectin [19].

In this context, this present study aimed to purify, characterize and reveal the complete amino acid sequence of a novel lectin from seeds of *C. microchaete*.

2. Materials and methods

2.1. Plant material

Fruits of *C. microchaete* were collected from Brejo Santo City in Salvaterra Town (Ceará State, Brazil). The mature seeds were removed from samaras and stored at room temperature for later use.

2.2. Lectin purification

The seeds were ground into a fine powder using a coffee mill, and the resulting flour was defatted using n-hexane and air-dried at room temperature. The soluble proteins contained in the powder were extracted in 100 mM Tris–HCl buffer pH 7.6 containing 150 mM of NaCl [1:10 (w/v)] at room temperature with continuous stirring overnight. The mixture was then centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant (crude protein extract) was applied to a mannose-Sepharose-4B (5 × 1 cm) column (GALAB Technologies GmbH, Geesthacht, Germany) equilibrated with 100 mM Tris–HCl buffer pH 7.6 containing 150 mM of NaCl. The unbound material was washed using equilibrium buffer, and the lectin was eluted with 100 mM of D-mannose in the same buffer. One milliliter fractions were manually collected, and absorbance was monitored at 280 nm. Lectin fractions were dialyzed against 100 mM acetic acid (to remove the D-mannose), followed by extensive dialysis against ultrapure water. Finally, fractions were freeze-dried and stored for subsequent characterization.

2.3. Hemagglutination activity and inhibition assays

Hemagglutinating activity tests of extract and pure lectin were performed by two-fold serial dilution method using rabbit and human erythrocytes that were either untreated or treated with the enzymes trypsin and papain, as previously described by Moreira and Perrone [27]. Hemagglutination values were expressed as hemagglutinating unit (H.U.) and defined as the minimal amount (mg) of protein per mL able to induce visible agglutination of cells.

Inhibition assay was carried out according to a standard procedure previously described by Ramos et al. [28]. Briefly, two-fold serial dilutions of D-glucose, D-mannose, D-galactose, D-arabinose, D-fructose, D-xylose, *N*-acetyl-D-glucosamine, α-methyl-mannoside, lactose, sucrose, as well as the glycoproteins porcine stomach mucin (PSM), fetuin and ovalbumin, were used at initial concentration of 100 mM (saccharides) and 1 mg mL^{-1} (glycoproteins).

2.4. Determination of protein content

To determine the protein content, the method described by Bradford [29] was applied by using bovine serum albumin (BSA) as standard.

2.5. Molecular mass determination

The relative molecular mass of the lectin was estimated by SDS-PAGE carried out as described by Laemmli [30] on slabs of 15% polyacrylamide in reduced and nonreduced conditions (presence and absence of β-mercaptoethanol 2%) using a Mini-Protean II apparatus (Bio-Rad, Milan, Italy). LMW-SDS marker kit (GE Healthcare, Buckinghamshire, UK) was used as the molecular weight markers.

The isotopic average molecular mass of lectin was determined using a hybrid Synapt HDMS mass spectrometer (Waters Corp., Milford, MA, USA). The lectin was solubilized in 50% acetonitrile (ACN) containing 0.1% formic acid (FA). After centrifugation, protein solution (10 pMol μL^{-1}) was infused at a flow rate of 1 $\mu L\,min^{-1}$ into a nanoelectrospray source coupled to a mass spectrometer. The instrument was calibrated with [Glu1]-Fibrinopeptide B fragments. Mass spectra were acquired by scanning at *m/z* range from 500 to 3,000 at 1 scan s^{-1}. The mass spectrometer was operated in positive mode, using a source temperature of 363 K and capillary voltage at 3.5 kV. Data collection and processing were controlled by Mass Lynx 4.1 software (Waters). Deconvolution of ESI mass spectra was performed using the MaxEnt 1 algorithm in the Mass Lynx software.

Moreover, the native mass of the lectin was estimated by gel filtration on a BioSuite TM 250 (7.8 × 300 mm–Waters) equilibrated with 50 mM Tris–HCl buffer (pH 7.6) containing 500 mM NaCl. A 0.5 mL sample containing 1 mg of pure lectin was loaded on the column and chromatographed at a flow rate of 500 $\mu L\,min^{-1}$. Chromatography was carried out in an ACQUITY UPLC System and calibrated with standard proteins: conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease (13.7 kDa) and aprotinin (6.5 kDa).

2.6. Glycoprotein detection and carbohydrate content determination

To determine whether the lectin is a glycoprotein, the lectin was subjected to 15% SDS-PAGE under denaturing conditions, as described above, and the gel was stained with the periodic acid-Schiff staining method [31]. Furthermore, the neutral sugar content of the lectin was estimated by the phenol-sulfuric acid method using D-glucose as a standard [32].

2.7. Effects of pH, temperature and ethylenediaminetetraacetic (EDTA) on lectin stability

The effect of pH, temperature and divalent ions on hemagglutinating activity was determined, as described by Sampaio et al. [33].

2.8. Primary structure determination by tandem mass spectrometry (MS/MS)

SDS-PAGE was performed, as described above. Gels used for digestion with Asp-N were supplemented with ethylene glycol diacrylate (EDA), as crosslinker, to a final concentration of 0.22%. EDA plugs were removed with NH_4OH treatment following the method of Bornemann et al. [34]. After staining, protein spots were excised and destained, as described by Shevchenko et al. [35].

Treated spots were subjected to digestion with the following enzymes: trypsin, chymotrypsin, Asp-N and pepsin. Digestions with trypsin and chymotrypsin were performed in ammonium bicarbonate 25 mM at 1:50 (w/w) (enzyme/substrate). Digestion with Asp-N was performed in ammonium bicarbonate 25 mM at 1:200 (w/w) (enzyme/substrate). Pepsin digestion was performed in HCl 100 mM at 1:50 (w/w) (enzyme/substrate). All digestions were maintained at 37 °C for 18 h.

The digestion was stopped with 2 μL of 2% formic acid (FA). The samples were washed four times with 5% FA in 50% ACN. The supernatants were collected and transferred to fresh tubes, pooled, vacuum-dried, solubilized in 20 μL of 0.1% FA, and centrifuged at 10,000 × *g* for 2 min. Two microliters of the peptide solution were loaded onto a C-18 (0.075 × 100 mm) nanocolumn coupled to a nanoAcquity system. The column was equilibrated with 0.1% FA and eluted with a 10–85% ACN gradient in 0.1% FA. The eluates were directly infused into a nanoelectrospray source. The mass spectrometer was operated in positive mode with a source temperature of 373 K, and a capillary voltage at 3.0 kV. LC-MS/MS was performed according to the data-dependent acquisition (DDA) method. The lock mass used in acquisition was *m*/*z* 785.84 ion of the [Glu1]-Fibrinopeptide B. The selected precursor ions were fragmented by collision-induced dissociation (CID) using argon as collision gas. All CID spectra were manually interpreted.

In-solution digestions were performed using RapiGest™ SF Surfactant (Waters Corp). One hundred μg of protein were solubilized in 100 μL of 0.1% RapiGest™. The mixture was centrifuged, and supernatant was submitted for digestion with trypsin, chymotrypsin and Asp-N, as described above. After incubation at 37 °C for 18 h, FA was added to the solution at a final concentration of 0.5%. Digestions were incubated at 37 °C for 1 h and centrifuged. Finally, two microliters of the peptide solution were loaded onto a C-18 nanocolumn, and LC-MS/MS experiments were conducted, as described above.

Also, both in-gel and in-solution digestions were analyzed by MALDI-Q-ToF, using a Synapt HDMS instrument operating at 20-kV accelerating voltage in the reflector V-mode. α-Cyano-4-hydroxycinnamic acid saturated in 50% acetonitrile and 0.1% trifluoracetic acid (TFA) was used as sample matrix.

2.9. Bioinformatics analysis

The sequence similarity of the peptides was evaluated online (http://www.ncbi.nlm.nih.gov/BLAST/) using the standard protein BLAST program available from the National Center for Biotechnology Information (NCBI).

Multiple alignment and secondary structure predictions were achieved using ESPript2.2 [36].

Table 1
Inhibitory effect of saccharides and glycoproteins on hemagglutinating activity of *Centrolobium microchaete* lectin.

Sugar and glycoproteins	MIC*
D-Mannose	3.1 mM
D-Glucose	6.25 mM
D-Galactose	NI**
N-acetyl-D-glucosamine	6.25 mM
α-Methyl-D-mannoside	3.1 mM
D-Xylose	NI
D-Fucose	NI
Sucrose	6.25 mM
Lactose	NI
Porcine stomach mucin	NI
Fetuin	NI
Ovalbumin	NI

* MIC, minimum inhibitory concentration.
** NI, sugar and glycoproteins not inhibitory until a concentration of 100 mM or 1 mg mL^{-1}, respectively.

3. Results

3.1. Hemagglutination and inhibition

Crude protein extract of seeds from *C. microchaete* showed hemagglutinating activity against rabbit erythrocytes treated with proteolytic enzymes or untreated. The extract presents H.U. of 32 for untreated erythrocytes and H.U. of 256 and 512 for erythrocytes treated with papain and trypsin, respectively. Interestingly, hemagglutination was not observed for human erythrocytes (untreated or treated with papain and trypsin). Hemagglutinating activity of the extract was strongly inhibited by D-mannose (3.1 mM) and α-methyl-D-mannoside (1.55 mM). Hemagglutination was also inhibited by D-glucose (6.25 mM), *N*-Acetyl-D-glucosamine (6.25 mM) and sucrose (12.5 mM); however, it was not inhibited by D-galactose, D-arabinose, D-fructose, D-xylose, lactose or the glycoproteins PSM, fetuin and ovalbumin (Table 1).

3.2. Purification of CML

As a result of the strong inhibition by D-mannose, the crude protein extract was applied in an affinity chromatography on mannose-Sepharose 4B column resulting in a bound fraction eluted with 100 mM of D-mannose that corresponded to pure lectin, termed *C. microchaete* lectin, or CML (Fig. 1A). The purified lectin at a concentration of 0.257 mg showed specific activity of 1992,22 H.U. mg^{-1}, whereas the crude extract showed 7.33 mg and 69.85 H.U. mg^{-1} for protein content and specific activity, respectively, showing an increase of specific activity by 28.5-fold in the pure lectin (Table 2).

3.3. Molecular mass and sugar content of CML

SDS-PAGE of CML, in reduced and nonreduced conditions (with and without β-mercaptoethanol), exhibited an electrophoretic profile consisting of a single band with an apparent molecular mass

Table 2
Purification of *Centrolobium microchaete* lectin.

Fraction	[a]Total protein (mg mL^{-1})	[b]Total HU	[c]Specific activity (H.U. mg^{-1})	Purification (fold)
Crude extract	7.33	512	69.85	1
CML	0.257	512	1992.22	28.5

[a] Protein content.
[b] Hemagglutinating activity expressed in hemagglutination units.
[c] Specific activity calculated as the ratio between hemagglutination units and protein content.

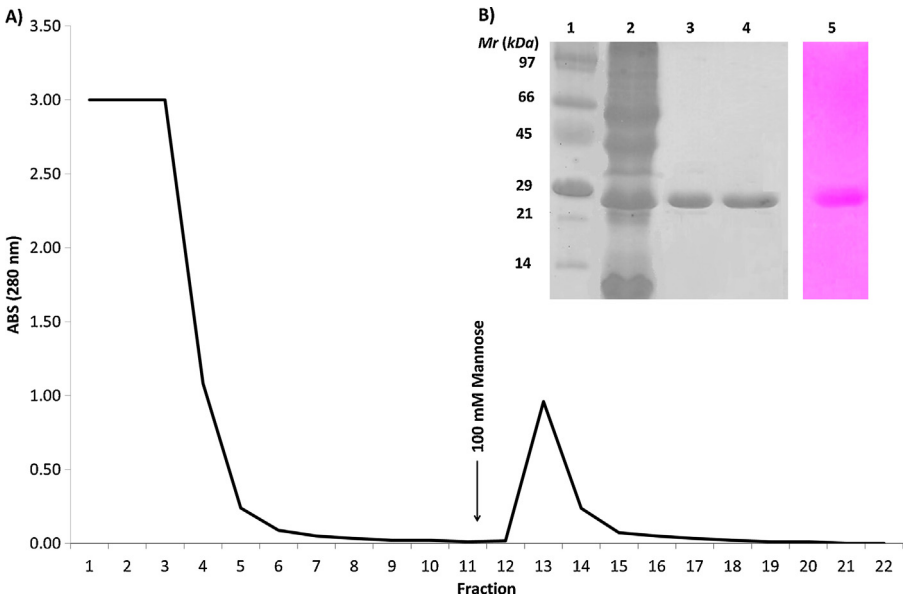

Fig. 1. Purification of CML. (A) Affinity chromatography on mannose-Sepharose 4B column of crude extract from *Centrolobium microchaete* seeds. Approximately 3 mL of crude extract were applied in the column (dimensions 5 × 2 cm) previously equilibrated with 100 mM Tris–HCl buffer pH 7.6 containing 150 mM of NaCl. The unbound fraction was eluted with equilibration solution (flow rate 1.0 mL min^{-1}). Arrow indicates the point at which 100 mM D-mannose was added. Fractions of 1 mL were collected and monitored by absorbance at 280 nm. (B) SDS-PAGE (15%) profile of CML. Lane 1: molecular mass markers (phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa); lane 2: crude extract; lane 3: CML (40 μg); lane 4: CML (40 μg) + β-mercaptoethanol; lane 5: CML (40 μg) stained with periodic acid-Schiff's reagent.

of 28 kDa, indicating that the sub-units are not linked by disulfide bonds (Fig. 1B). ESI-MS analysis of CML preparations revealed three ion series. Three distinct molecular mass specimens were identified with 27,224 ± 2 Da, 27,338 ± 2 Da, and 28,409 ± 2 Da, confirming the results obtained by SDS-PAGE. It should be noted that slight variations were found around these masses, indicating the presence of adducts (Fig. 2). The analytical gel filtration chromatography of CML, under nondenaturing conditions, showed a single sharp symmetrical peak, confirming lectin purity, with molecular mass at approximately 42 kDa (Fig. 3).

To verify glycosylation in CML, SDS-PAGE was performed, followed by Schiff staining. CML was stained with Schiff's reagent, implying that the lectin is a glycoprotein (Fig. 1B). Further confirming this result, data from the phenol-sulfuric acid assay showed that CML is a composite of approximately 1.5% carbohydrates by weight (data not shown).

3.4. Effects of temperature, pH, and divalent cations on the hemagglutinating activity of CML

CML showed thermal stability, maintaining its hemagglutinating activity, even after incubation up to 50 °C for 1 h. However, activity was drastically reduced at 60 °C and completely lost at temperatures greater than 80 °C (Fig. 4A). The hemagglutinating activity of CML was maintained over a wide range of pH values. Stability was more apparent at pH 7.0 and 8.0, but it was reduced in acidic and alkaline pH (Fig. 4B). CML was submitted to dialysis against EDTA and NaCl, after which its hemagglutinating activity

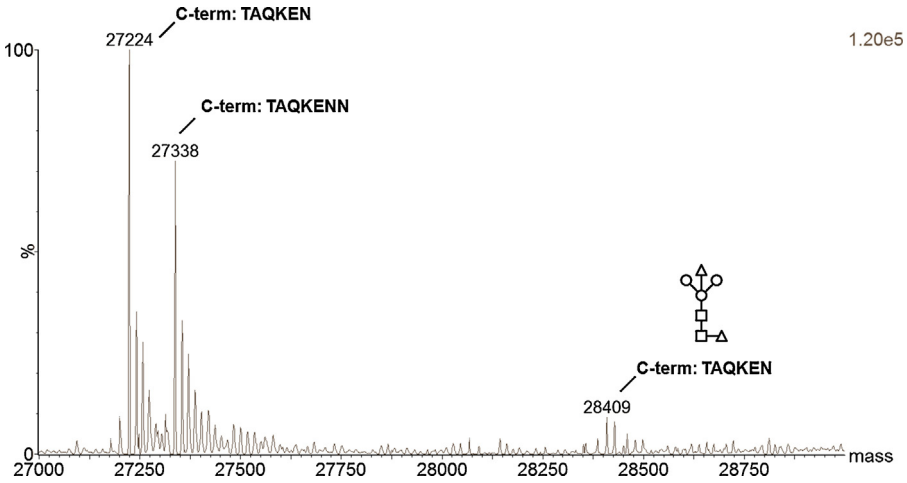

Fig. 2. Molecular mass determination of CML acquired by ESI-MS. Deconvoluted mass spectra showing the average mass of CML-1, CML-2 and CML-1 with glycan $HexNAc_2Hex_3DeoxyHex_2$ (□ - HexNAc; ○ - Hex; △ - DeoxyHex).

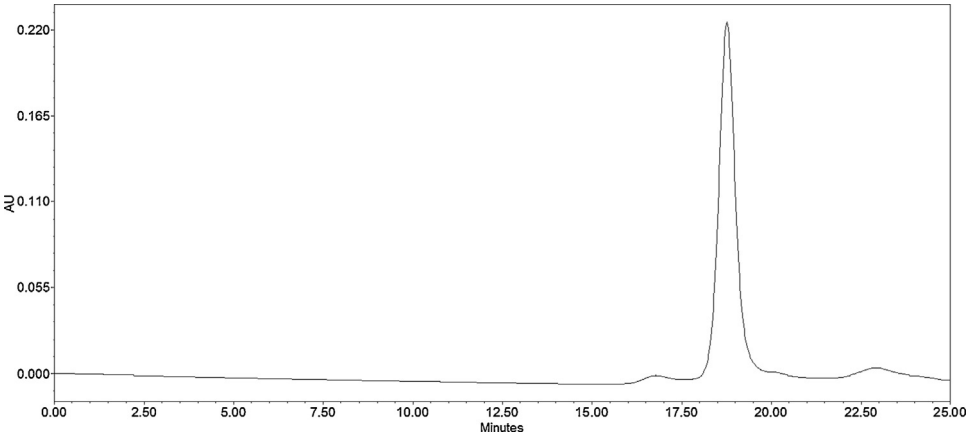

Fig. 3. Size exclusion profile of CML in BioSuite 250.5 μm HR SEC column. Absorbance was monitored at 280 nm.

was reduced and then partially recovered after the addition of $CaCl_2$ and $MnCl_2$ (data not shown), suggesting that CML is a metalloprotein and that its activity is dependent on these divalent cations.

3.5. Primary structure of CML

Overlapping of peptides originated by proteolysis was used for determination of amino acid sequence of CML (Fig. 5). Two distinct forms in CML preparations were sequenced. The two isolectins, CML-1 and CML-2, diverge on its C-terminal regions. CML-2 possesses one more residue (Asn) in comparison to CML-1. Their primary structures were found to consist of 246 and 247 residues, totaling 27,224 ± 2 Da and 27,338 ± 2 Da, respectively. These values are good agreement with molecular mass determined by MS.

Among the 247 amino acids of CML-2, two residues (^{75}SF^{76}) could not be identified by MS/MS, but they were identified by their similarity with other legume lectins. Furthermore, two glycosylated peptides were found: $[M+3H]^{3+}$ ion precursor of SPKTAQNESANQVLAVEF peptide at *m/z* 834.7158 and $[M+2H]^{2+}$ ion precursor of PKTAQNESANQV peptide at *m/z* 919.8988. These peptides showed differences in their calculated molecular mass, suggesting the presence of two *N*-acetylhexosamine residues and one hexose residue in the peptide at *m/z* 834.7158, as well as two *N*-acetylhexosamine residues and one deoxyhexose residue in the peptide at *m*/*z* 919.8988.

A databank search revealed high similarity between CML and such legume lectins as PeLA (*Platypodium elegans* lectin A), PaL (*Pterocarpus angolensis* lectin), *Pterocarous rotundifolius* lectin (gene) and *Arachis hypogaea* mannose-binding lectin (Fig. 6).

4. Discussion

Plants from the Dalbergiae represent a tribe of the leguminous family which remains understudied and poorly characterized in the context of new lectins. Therefore, this study report the purification and characterization of a novel Dalbergiae tribe legume lectin, CML. CML was purified through a single affinity chromatography step in mannose-Sepharose 4B column. Since lectins characteristically bind with high specificity to sugar, affinity chromatography is a very useful technique for the purification of these proteins, as described previously for other plant lectins [18,37–40].

CML preferentially agglutinated enzyme-treated rabbit erythrocytes. In fact, some works have demonstrated increased hemagglutinating activity after treatment of cells with proteolytic enzymes [18,19]. Moreover, the hemagglutinating activity was inhibited by D-mannose, D-Glucose, *N*-acetyl-D-glucosamine, α-Methyl-D-mannoside and sucrose. Similar to other legume lectins, CML presents preference for sugars containing substituents with greater hydrophobicity in carbons 1 and 2, thus increasing the affinity of this lectin for this sugars [18,19,28,37–39]. Interestingly, despite the presence of an acetyl group in carbon 2 of the *N*-acetyl-D-glucosamine, no difference of affinity was found when compared with D-glucose. However, the presence of a methyl group in carbon 1 of methyl-D-mannoside significantly increased the affinity of the lectin when compared with D-mannose. Like CML, other Dalbergiae

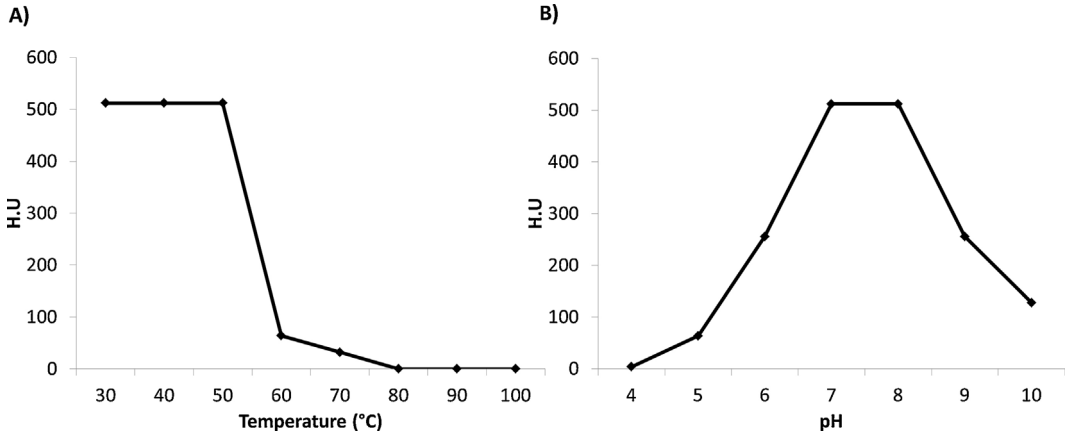

Fig. 4. Physicochemical properties of CML. The effect of pH (A) and temperature (B) on hemagglutinating activity of CML.

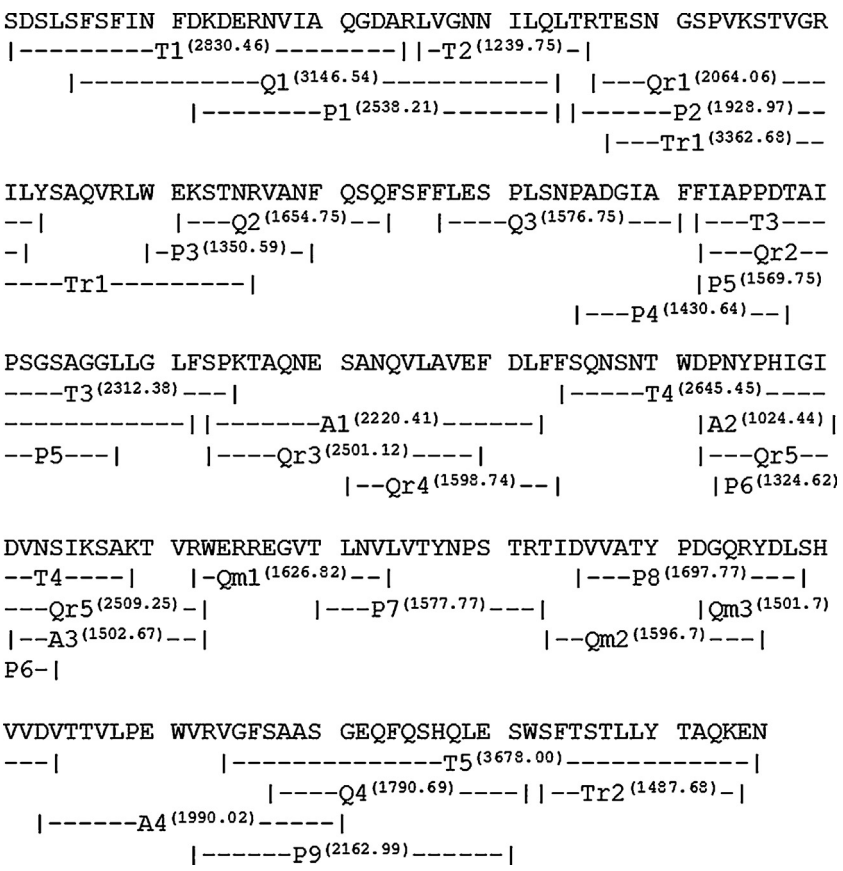

Fig. 5. Amino acid sequence of CML. Peptides originated by different digestions are represented by T (trypsin in gel), Tr (trypsin RapiGest), Q (chymotrypsin in gel), Qr (chymotrypsin RapiGest), Qm (chymotrypsin in gel by MALDI), P (pepsin in gel) and A (Asp-N RapiGest).

lectins have specificity for mannose and its derivatives, i.e., PAL, PeLA, *P. floribundum* (PFL) and *C. tometosum* lectin (CTL) [15,18–20].

The SDS-PAGE profile of CML showed a single band with a molecular mass of approximately 28 kDa, and analysis by ESI-MS showed CML with three distinct molecular masses of approximately 28 kDa. Curiously, the molecular mass of native CML, as estimated using gel filtration, is 42 kDa, suggesting that CML is a homodimer. In fact, legume lectins are generally derived from protomers of approximately 30 kDa and are homodimers or homotetramers, with the tetramers being dimers of dimers [3,11]. Other Dalbergiae lectins also exist in dimeric form, as shown by the crystal structures of PAL and PeLA [15,20]. Moreover, analytic gel filtration chromatography showed that PFL and CTL are also dimeric proteins [18,19].

CML was found to be a thermostable lectin similar to the other legume lectins [12,18,19,37,38]. CML activity was also maintained in spite of wide pH variation, with stability being more apparent between pH 7.0 and 8.0, suggesting that CML is sensitive to acidic and alkaline buffers.

Several strategies were used to achieve full coverage of CML amino acid sequence. First, a bottom-up strategy was chosen since it is fast and sensitive, but only about 85% of coverage was obtained. Apart from the conventional SDS-PAGE, gels containing a mix of bis acrylamide and EDA were used to that Asp-N endopeptidase were able to digest CML.

An alternative approach was employed: in solution digestion using RapiGest, a surfactant. Some exclusive peptides were found using this approach, mainly when chymotrypsin was used. Finally, MALDI was employed to confirm the amino acid sequence coverage. MALDI is perhaps the most efficient method of peptide ionization [41]; therefore, peptides not observable using ESI were analyzed by MALDI. For instance, the $[M+1H]^{1+}$ ion precursor of RWERREGVTLNVL peptide at *m/z* 1627.8361 was only found when chymotrypsin digestions were analyzed by MALDI.

CML sequence shows homology with other mannose-specific legume lectins, including PeLA (87%), PAL (75%), *Pterocarpus rotundifolius* lectin (PRL) (80%) and *Arachis hypogaea* mannose-binding lectin (67%). Similar to PAL and PeLA, CML presents a region commonly described as a carbohydrate binding site [15,20], indicating that CML recognizes sugars in a manner similar to that displayed by PeLA and PAL.

Other lectins from the Dalbergiae tribe have been sequenced, but they have shown low homology with CML. For instance, the lectins isolated from the genus *Vatairea* present specificity for galactose and its derivatives and exhibit a complex post- translational process [13,21]. Obviously CML does not bind to galactose, and no evidence revealed that CML undergoes lectin processing similar to that of *Vatairea*.

This Dalbergiae dataset suggests the existence of at least two subfamilies of lectins that could be observed in this tribe. The first subfamily includes PAL, PRL, PeLA, CTL CML and PFL. These lectins shared specificity for mannose, one glycosylation site, a short signal peptide, but no evidence of proteolytic processing in internal regions; therefore, just one polypeptide chain is observed in these proteins [15,18,19,20]. The second subfamily presents lectins from the genus *Vatairea* that share the following characteristics: specificity for galactose and derivatives, two glycosylation sites, and at least three polypeptide chains (α, β and γ). Furthermore, a complex post-translational modification that originates distinct polypeptide chains could be observed in the lectins from the second subfamily [13,21].

Preparations of purified lectins from the *Dalbergiae* subtribe are, in general, composed of diverse isoforms [13,18,21]. In CML, two

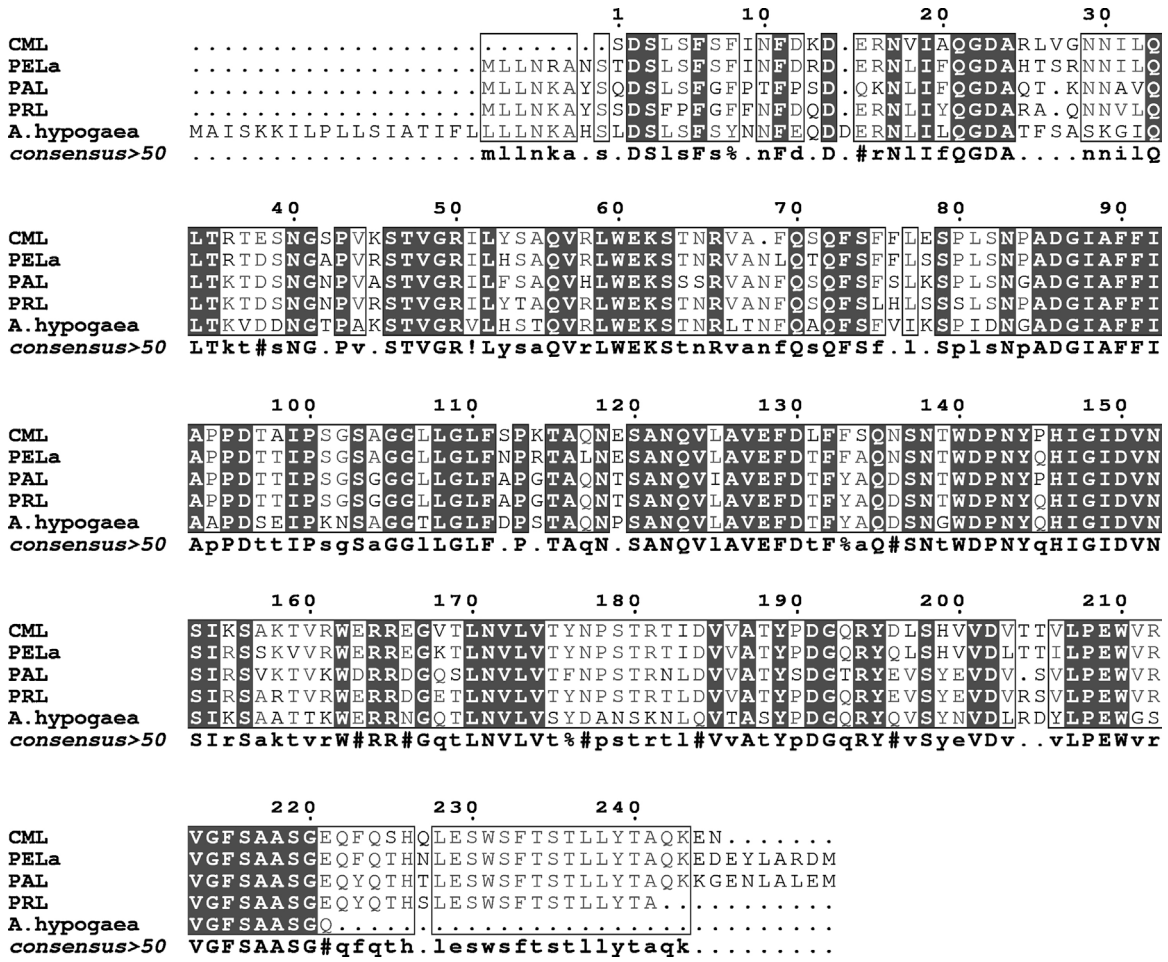

Fig. 6. Multiple alignments of the CML sequence with sequences from other legume lectins using the webESPript program. The sequence data were obtained from the Entrez protein sequence database. ID numbers are as follows: AEK69351.1 (*P. elegans* lectin), CAD19804 (*P. angolensis* lectin), AAT57665.1 (*P. rotundifolius* lectin) and AAA74572.1 (*A. hypogaea* lectin).

isolectins were identified. Specifically, CML-1 and CML-2 diverge in lectin chain lengths of 246 and 247 residues, respectively.

C-terminal region of legume lectins generally exhibit proteolytic processing [42]. Although this processing is not clearly understood, Chrispeels and Raikhel [43] suggest that plant proteins are targeted to vacuoles by short peptide domains, including short peptides on C- and N-terminal regions. Since *Dalbergiae* lectin sequences, as determined by cDNA, exhibit a C- and N-terminal extension in comparison with CML, it is likely that CML undergoes proteolytic processing in both regions.

Sugar analysis showed that CML is a glycoprotein containing approximately 1.5% carbohydrate, an amount very similar to that of PFL and CTL, approximately 2 and 1.7%, respectively [18,19]. Dalbergiae lectins generally possess glycosylation sites and hybrid glycans [13,15,18,21]. CML has one glycosylation site and, very likely, one hybrid glycan, since peptides containing HexNAc, Hex and Deoxy-Hex were identified. Also, ESI-MS data indicated three molecular mass specimens in CML preparations. Two of them are related to the isoforms that diverge in one amino acid: CML-1 and CML-2, with mass difference of 114 Da (Asn residue on C-terminal). The third molecular mass observed in deconvoluted spectra, 28,409 ± 2 Da, may represent the glycosylated form of CML-1, containing the hybrid glycan $HexNAc_2Hex_3DeoxyHex_2$ with average molecular mass of 1,185 Da. This glycan is similar to the one found on lectins from the genus *Vatairea*, differing only by the presence of a DeoxyHexose residue in substitution of pentose residue (Xylose) [13,21]. CML, like other legume lectins, is a metalloprotein, and its metal binding site is conserved in comparison to PAL and PeLA [15,20].

In summary, this study reported the purification and characterization of a novel mannose-binding lectin of *C. microchaete* seeds (CML). CML is a metal-dependent glycoprotein that showed a molecular mass of approximately 28 kDa containing approximately 1.5% carbohydrate and was specifically inhibited by D-mannose, α-methyl-D-mannoside, D-glucose, N-Acetyl-D-glucosamine and sucrose. Furthermore, CML showed sequence similarity with other legume lectins and has one glycan similar to that found on lectins from the genus *Vatairea*. Moreover, the lectin is a mixture of two isolectins (CML-1 and CML-2) that diverge on its C-terminal regions.

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